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CCN3 (NOV) Is a Novel Angiogenic Regulator of the CCN Protein Family*

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CCN3 (NOV) is a matricellular protein of the CCN family, which also includes CCN1 (CYR61), CCN2 (CTGF), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3). During development, CCN3 is expressed widely in derivatives of all three germ layers, and high levels of expression are observed in smooth muscle cells of the arterial vessel wall. Altered expression of CCN3 has been observed in a variety of tumors, including hepatocellular carcinomas, Wilm's tumors, Ewing's sarcomas, gliomas, rhabdomyosarcomas, and adrenocortical carcinomas. To understand its biological functions, we have investigated the activities of purified recombinant CCN3. We show that in endothelial cells, CCN3 supports cell adhesion, induces directed cell migration (chemotaxis), and promotes cell survival. Mechanistically, CCN3 supports human umbilical vein endothelial cell adhesion through multiple cell surface receptors, including integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and heparan sulfate proteoglycans. In contrast, CCN3-induced cell migration is dependent on integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, whereas $\alpha_6\beta_1$ does not play a role in this process. Although CCN3 does not contain a RGD sequence, it binds directly to immobilized integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, with half-maximal binding occurring at 10 nM and 50 nM CCN3, respectively. Furthermore, CCN3 induces neovascularization when implanted in rat cornea, demonstrating that it is a novel angiogenic inducer. Together, these findings show that CCN3 is a ligand of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, acts directly upon endothelial cells to stimulate pro-angiogenic activities, and induces angiogenesis *in vivo*.

CCN3[1] (NOV, nephroblastoma overexpressed) was originally identified as an aberrantly expressed gene in avian nephroblastomas induced by myeloblastosis-associated virus (1, 2). It is a member of the CCN[1] family, which also includes CCN1 (CYR61), CCN2 (CTGF), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3) (3, 4). Inasmuch as these cysteine-rich, secreted proteins are associated with the ECM but serve regulatory rather than structural functions, they can be considered matricellular proteins (5). Members of the CCN family are composed of an N-terminal secretory signal peptide followed by four conserved domains with homology to insulin-like growth factor binding protein, von Willebrand factor type C repeat, thrombospondin type 1 repeat, and a C-terminal domain (CT) with heparin-binding motifs and sequence similarity to the C termini of von Willebrand factor and mucin (6). In keeping with their homology to ECM proteins and localization to the ECM, several CCN proteins have been shown to support cell adhesion, induce focal adhesion complexes, and stimulate adhesive signaling (7–9). Among members of the CCN family, CCN1 and CCN2 have been most extensively characterized. Both proteins stimulate cell migration, promote cell survival, and augment growth factor-induced mitogenesis (10–14). Both proteins are known to induce angiogenesis and chondrogenesis (12, 15–18). Although CCN proteins do not contain a RGD sequence motif, both CCN1 and CCN2 are direct ligands of multiple integrin receptors, which mediate many of their activities (11, 13, 14, 19–22). Targeted disruption of the *CCN1* gene in mice resulted in embryonic lethality due to vascular defects (23), whereas *CCN2*-null mice die perinatally due to respiratory failure as a consequence of skeletal malformation (18). These findings indicate that members of the CCN family serve essential and non-redundant functions during development.

Relatively little is known about the functions of CCN3. During embryonic development, CCN3 is widely expressed in derivatives of all three germ layers, with high levels of expression in skeletal muscle, smooth muscle of vessel walls, the nervous system, adrenal cortex, and differentiating chondrocytes (24–27). In the adult, CCN3 expression is high in the arterial vessel wall, and the expression pattern changes following vascular injury (28). CCN3 interacts with the epidermal growth factor-like domain of Notch1 and positively regulate Notch signaling (29). In addition, CCN3 interacts with fibulin in a yeast two-hybrid assay and may regulate calcium signaling (30, 31). Altered expression of CCN3 has been observed in a variety of tumors, including hepatocellular carcinomas, Wilm's tumors, Ewing's sarcomas, gliomas, rhabdomyosarcomas, and adrenocortical carcinomas (27, 32–34).

In contrast to *CCN1* and *CCN2*, which are immediate-early genes transcriptionally activated by mitogenic growth factors in fibroblasts and are repressed under conditions of growth arrest (35–37), *CCN3* is repressed by growth factors but induced by serum deprivation or contact inhibition (38, 39). Thus, it has been hypothesized that CCN3 may serve as an antago-

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[1] The abbreviations used are: CCN, cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed; BACEC, bovine adrenal capillary endothelial cell; BSA, bovine serum albumin; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin; GST, glutathione *S*-transferase; HUVEC, human umbilical vein endothelial cell; IgG, immunoglobulin G; LN, laminin; mAb, monoclonal antibody; TUNEL, *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling; VN, vitronectin; nt, nucleotides.

nist to CCN1 and CCN2, and play antithetical roles in similar biological processes. Since CCN1 and CCN2 have been shown to be angiogenic inducers (12, 16, 17), we speculated that CCN3 might work as an angiogenic inhibitor. In this study, we show that purified recombinant CCN3 interacts with multiple integrin receptors and binds directly to integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Contrary to expectation, CCN3 is pro-angiogenic in endothelial cells, supporting cell adhesion and promoting cell migration and survival. Furthermore, CCN3 induces neovascularization in a corneal micropocket assay. These results identify CCN3 as a novel angiogenic factor and suggest its possible functions in development and tumor growth.

MATERIALS AND METHODS

Cell Culture—HUVECs were grown as described by the supplier (Cascade Biologics, Inc., Portland, OR) and used before passage 16. BACECs (a generous gift from Dr. Judah Folkman, Harvard Medical School) were grown in plates coated with 1.5% gelatin (Difco Laboratories, Detroit, MI) and maintained in Dulbecco's modified Eagle's medium (DMEM, JRH Bioscience, Lenexa, KS) supplemented with 10% fetal bovine serum (Intergene, Purchase, NY) and 3 ng/ml bFGF (Invitrogen, Carlsbad, CA); they were used before passage 16.

Antibodies, Peptides, and Reagents—Function-blocking mAbs against various integrins were purchased from Chemicon (Temecula, CA) [AV1 (anti-α_v), LM609 (anti-$\alpha_v\beta_3$), and JBS5 (anti-$\alpha_5\beta_1$)], Beckman-Coulter, Inc. (Fullerton, CA) [NKI-GoH3 (anti-α_6), and SAM-1 (anti-α_5)], and Invitrogen [P4C10 (anti-β_1)]. Normal mouse IgG was from Zymed Laboratories, Inc. (South San Francisco, CA) and normal rabbit serum was from Sigma-Aldrich. GRGDSP and GRGESP peptides were purchased from Invitrogen. RGDS and RGES peptides were from American Peptide Company, Inc. (Sunnyvale, CA). Heparin (sodium salt, from porcine intestinal mucosa) was from Sigma-Aldrich. bFGF, FN, VN, and LN were obtained from Invitrogen. Purified integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ were from Chemicon.

Purification of Recombinant CCN3—Human *CCN3* cDNA was constructed by ligation of a 5′ (nt 72–654, GenBank™ X96584) and 3′ (nt 654–1653) fragments, and the resulting full-length cDNA was cloned into pKS+ and verified by sequencing. The 5′ fragment (nt 72–654) was obtained by reverse transcriptase-polymerase chain reaction using total RNA isolated from serum-starved human skin fibroblasts using the primer set 5′-AGCAGTGCCAATCTACAGC-3′ and 5′-CAGCATCTCACATTGACGG-3′. The RT-PCR product was digested with *Sph*I and *Sty*I to yield a fragment containing nt 72–654. The 3′ fragment (nt 654–1653) was generated by restriction digestion of IMAGE clone 49415 (human neonatal brain, nt 590–1653) with *Sty*I and *Xba*I. To produce recombinant CCN3 protein, the full-length *CCN3* cDNA was cloned into the baculovirus expression vector pBlueBac 4.5 (Invitrogen). The vector was modified to encode an enterokinase histidine tag linked to the C terminus of CCN3 in a manner similar to that previously described for expression of CCN1 (21). CCN3 was produced in serum-free baculovirus expression system using High Five insect cells as described (21). Briefly, High Five cells were maintained in serum-free EX-CELL 400 medium (JRH Bioscience) at 27 °C and infected at a multiplicity of infection of 10. Conditioned medium was collected at 38 h postinfection, adjusted to 20 mM sodium phosphate, and applied to a Sepharose SP (Sigma-Aldrich) column at 4 °C. After washing with a 20 mM sodium phosphate buffer (pH 6.0) containing 300 mM NaCl, bound proteins were eluted with a linear gradient of NaCl (0.4–1 M) in phosphate buffer. Fractions containing CCN3 as judged by SDS-PAGE were pooled and further purified on a nickel-agarose column as described (21). Fractions were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining and immunoblotting (Fig. 1).

Preparation of Antisera and Affinity-purified Antibodies—The second domain of CCN3 (von Willebrand type C repeat) and the central variable region were cloned separately as glutathione *S*-transferase (GST) fusion proteins and used as antigens to immunize New Zealand White rabbits. DNA fragments were generated by polymerase chain reactions using primers sets 5′-CGCGGATCCGCGGTAGAGGGAGATAACTGTG-3′ and 5′-CCGGAATTCAGCTGCAAGGGTAAGGCCTCC-3′ (encoding amino acids 104–188), and 5′-GATGAGGAGGATTCACTGGGA-3 and 5′-AATGCAGTTGACACTTGAG-3′ (encoding amino acids 176–207). To facilitate cloning, the forward primers start with a *Bam*HI site, and the reverse primers end with an *Eco*RI site. The resulting cDNA fragments were cloned directionally into the PGEX-2T vector (Amersham Biosciences) and confirmed by sequence analysis. The GST fusion proteins were purified on a glutathione *S* Sepharose column and used as antigens. Antisera and affinity-purified antibodies were produced according to standard protocols (40). IgG was purified from antisera using protein A column chromatography (Pierce Biotechnology). For affinity purification, antisera were first passed through a GST column to remove antibodies against GST and then purified through a GST-CCN3 (VWC domain)-affinity column. Anti-CCN3 antibodies did not cross-react with CCN1 or VN (data not shown) by ELISA.

Cell Adhesion Assay—Cell adhesion assays were performed essentially as described (7). Briefly, test proteins were diluted in phosphate-buffered saline and coated onto 96-well microtiter plates (50 μl per well) with incubation at 4 °C for 16 h. Wells were rinsed with phosphate-buffered saline and blocked with 1% BSA at room temperature for 1 h. To test for specificity, affinity-purified anti-CCN3 antibodies or normal rabbit IgG was added to the wells and incubated for 1 h at 37 °C prior to plating of cells. HUVECs were harvested in phosphate-buffered saline containing 2.5 mM EDTA, washed, and resuspended at 2.5×10^5 cells/ml in serum-free Iscove's modified Dulbecco's medium containing 1% BSA. Where indicated, cells were mixed with either EDTA, Ca^{2+}, Mg^{2+}, peptides, or heparin prior to plating or incubated with antibodies for 1 h at room temperature prior to plating. Cell suspension (50 μl) was added to each well, and adherent cells were fixed in 10% formalin after 30 min incubation at 37 °C. Cells were stained with methylene blue, and adhesion was quantified by dye extraction and measurement of absorbance at 620 nm (7).

Solid Phase Integrin Binding Assay—The binding of CCN3 to purified integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ was measured by ELISA as described previously with modifications (19). For CCN3 binding to immobilized integrin, microtiter wells (Immulon 2, Dynatech Laboratories, Chantilly, VA) were coated with purified integrin (1 μg/ml) and incubated at 4 °C overnight. The wells were washed with buffer (20 mM Hepes, pH 7.5 containing 150 mM NaCl, 0.1 mM $CaCl_2$, and 2 mM $MgCl_2$) and blocked with 1% heat-inactivated BSA for 2 h at room temperature. Soluble CCN3 was added and allowed to bind at 4 °C for 16 h; bound ligand was detected using affinity-purified anti-CCN3 antibodies (1:1000). For integrin binding to immobilized ligands, microtiter wells were coated with 10 μg/ml CCN3, 10 μg/ml FN, or 1 μg/ml VN as described above. Where indicated, coated wells were preincubated with affinity-purified anti-CCN3 antibodies or normal rabbit serum for 2 h at room temperature. After washing, purified integrin $\alpha_v\beta_3$ or $\alpha_5\beta_1$ (1 μg/ml in buffer with 25 mM octylglucoside) was added and incubated overnight at 4 °C. Where indicated, soluble integrin was either mixed with EDTA, Mg^{2+}, or peptides prior to plating, or incubated with function-blocking monoclonal antibodies for 30 min at 4 °C prior to plating. After washing, bound integrins were detected with polyclonal anti-integrin α_v (AB1930) or α_5 antibodies (AB1926)(Chemicon). When blocking with anti-CCN3 antibodies, mAbs against integrin α_v (P3G8) or β_1 (HUTS-4)(Chemicon) were used to detect integrin binding. After washing, wells were incubated with horseradish peroxidase-conjugated secondary antibody (1:2500), and color reaction was developed using a horseradish peroxidase immunoassay kit (Zymed Laboratories, Inc.) with absorbance measured at 420 nm.

Cell Migration Assay—A 48-well modified Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD) was used to assay cell migration as described with modifications (16). BACECs were harvested with trypsin, washed, and resuspended at 5×10^5 cells/ml in Dulbecco's modified Eagle's medium containing 0.1% BSA. Cells were loaded into wells of the lower chamber; the wells were then covered with a gelatinized polycarbonate filter (5 μm pore diameter, Nuclepore) followed by the upper chamber. Where indicated, cells were either mixed with peptides or incubated with antibodies (1 h at room temperature) prior to loading. The chamber was inverted to allow cells to attach (2 h at 37 °C) and re-inverted, and test proteins, diluted in Dulbecco's modified Eagle's medium containing 0.1% BSA, were added to the top chamber. Where indicated, CCN3 and FN were preincubated with anti-CCN3 antibodies for 1 h prior to loading. After a 4-h incubation at 37 °C, the membrane was removed and stained using a Diff-Quik Kit (Dade-Behring, Deerfield, IL). Cell migration was monitored by counting the total number of cells migrated in 10 randomly selected microscope fields at ×400 magnification.

Measurement of Apoptosis and DNA Synthesis—Apoptosis and mitogenesis was assessed largely as described (14). To measure apoptosis, HUVECs were starved for 16 h, harvested, and resuspended in serum-free medium containing 0.5% BSA. Coverslips were coated overnight at 4 °C with 20 μg/ml mouse LN (ultrapure grade; Becton-Dickinson Biosciences, Bedford, MA) and blocked with 1% heat-inactivated BSA. Cells were plated on LN-coated coverslips at 10,000 cells/cm^2 and allowed to attach for 4 h at 37 °C. Soluble CCN3, serum (20%) or GRGDSP peptide (50 μM) was added, and incubation continued for

another 16 h at 37 °C. Where indicated, CCN3 was incubated with anti-CCN3 antibodies for 1 h at room temperature prior to addition to cells. After incubation, cells were fixed with 4% paraformeldahyde (pH 7.4) and apoptosis was detected by TUNEL assay using the *in situ* cell death detection kit POD (Roche Applied Science, Indianapolis, IN). Cells were lightly stained with hematoxylin and apoptotic nuclei counted. A total of 500 cells were counted from random fields in each coverslip, and the number of apoptotic cells was represented as a percentage of the total cells counted. To assess proliferation, cells were grown as described above except that 10 μM bromodeoxyuridine (BrdUrd) was included in the medium for 16 h in the presence or absence of CCN3. BrdU incorporation was detected using the BrdUrd staining kit (Calbiochem Novabiochem Corp., San Diego, CA).

Rat Corneal Pocket Angiogenesis Assay—CCN3-induced neovascularization was examined *in vivo* by implanting Hydron pellets, formulated with test substances, into rat corneas essentially as described (16). Briefly, male Sprague-Dawley rats were anesthetized and Hydron pellets (Interferon Sciences, Inc., New Brunswick, NJ) containing test substances were implanted into micropockets made in the corneal stroma 1 to 1.5 mm from the corneal limbus. Where indicated, CCN3 and bFGF were incubated with anti-CCN3 antibodies for 1 h at room temperature prior to being incorporated into the Hydron pellet. 7 days postimplantation, rats were perfused with India ink with heparin (100-U bolus), and neovascularization was examined and scored.

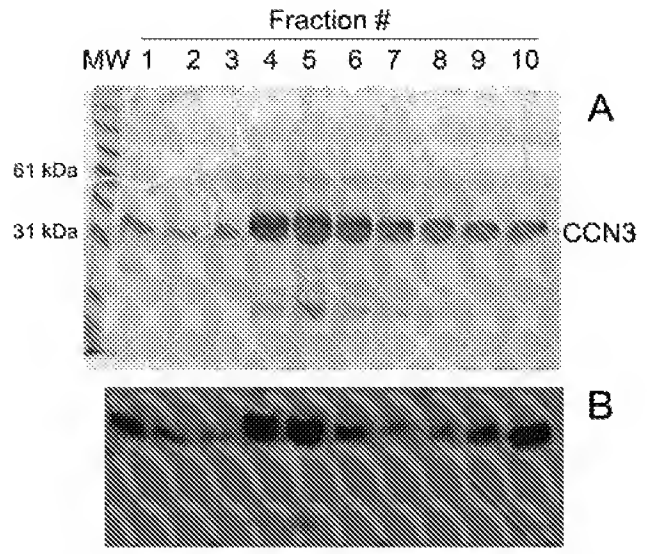

FIG. 1. **Purification of CCN3.** *A*, fractions (30 μl per lane) from nickel-agarose column chromatography were separated by 10% SDS-PAGE, followed by staining with Coomassie Brilliant Blue. *MW*, molecular weight marker. *B*, same fractions as in *A* (10 μl per lane) were analyzed on SDS-PAGE followed by immunoblotting with anti-CCN3 antibodies.

RESULTS

CCN3 Mediates Endothelial Cell Adhesion through Integrins $\alpha_v\beta_3$, $\alpha_6\beta_1$, and $\alpha_5\beta_1$—To examine the pro-angiogenic activities of CCN3, we purified recombinant human CCN3 using a baculovirus expression system (Fig. 1). We first tested its ability to support endothelial cell adhesion, and found that immobilized CCN3 was able to support HUVEC adhesion in a dose-dependent and saturable manner (Fig. 2*A*). Incubation of CCN3-coated surfaces with affinity-purified anti-CCN3 antibodies prior to plating cells abolished adhesion, whereas incubation of VN-coated surfaces with the same antibodies had no effect (Fig. 2*B*). These results confirmed that the CCN3 protein indeed supports HUVEC adhesion.

CCN1 and CCN2 are known to mediate HUVEC adhesion through integrin $\alpha_v\beta_3$ (19) and fibroblast adhesion through integrin $\alpha_6\beta_1$ and heparan sulfate proteoglycans (8, 20). HUVEC adhesion to CCN3, as well as to FN or VN, was inhibited by EDTA and restored by the addition of Mg^{2+} or Ca^{2+} (Fig. 2*C*). These results are consistent with the notion that divalent cation-dependent cell adhesion molecules, such as integrins, mediate cell adhesion to CCN3 (Fig. 2*C*). To define the specific integrins that might be involved, we investigated the inhibitory effect of RGD peptide. The GRGDSP peptide, but

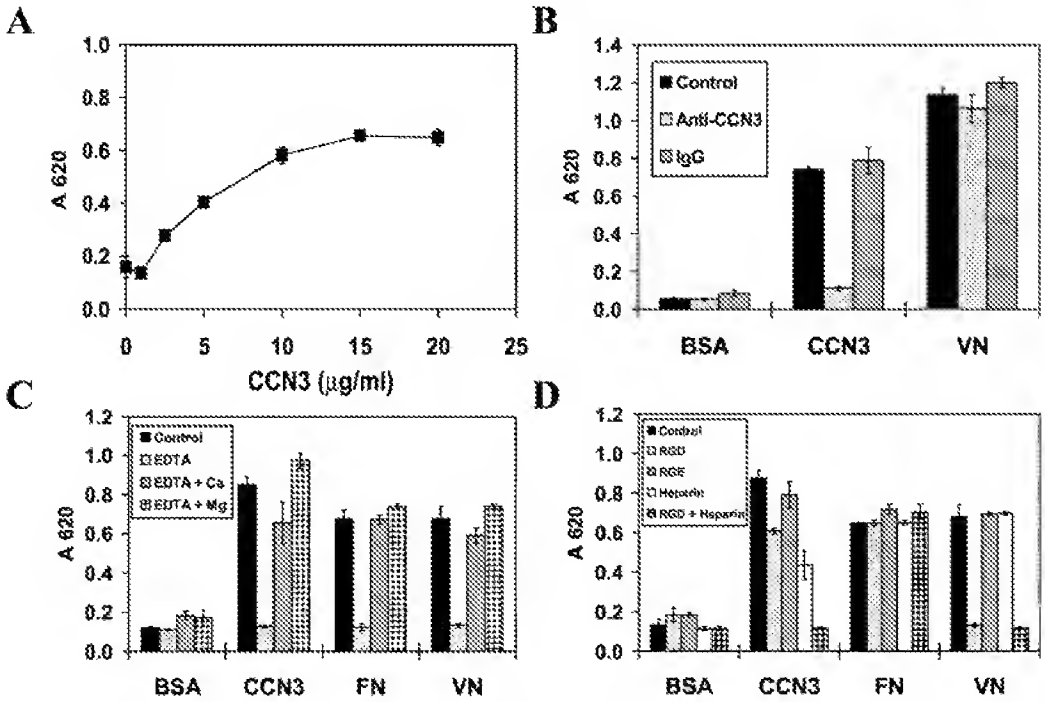

FIG. 2. **Adhesion of HUVECs to CCN3.** *A*, HUVECs were plated in microtiter wells coated with the indicated amount of CCN3. After incubation at 37 °C for 30 min, adherent cells were fixed, stained with methylene blue, and extracted dye was quantified by absorbance at 620 nm. *B*, microtiter wells were coated with BSA, 12 μg/ml CCN3, or 0.5 μg/ml VN, and where indicated, they were blocked with affinity-purified anti-CCN3 antibodies or normal rabbit IgG for 1 h at 37 °C prior to the addition of HUVECs. *C*, cells were plated on microtiter wells coated with 12 μg/ml CCN3, 3 μg/ml FN, or 0.5 μg/ml VN. EDTA (10 mM) was added alone or in combination with Ca^{2+} or Mg^{2+} (20 mM) as indicated. *D*, cells were incubated with GRGDSP, GRGESP peptides (0.2 mM), heparin (1 μg/ml); or in combination prior to plating on microtiter wells coated with CCN3, FN, or VN. Data shown are mean ± S.D. of triplicate determinations and are representative of three experiments.

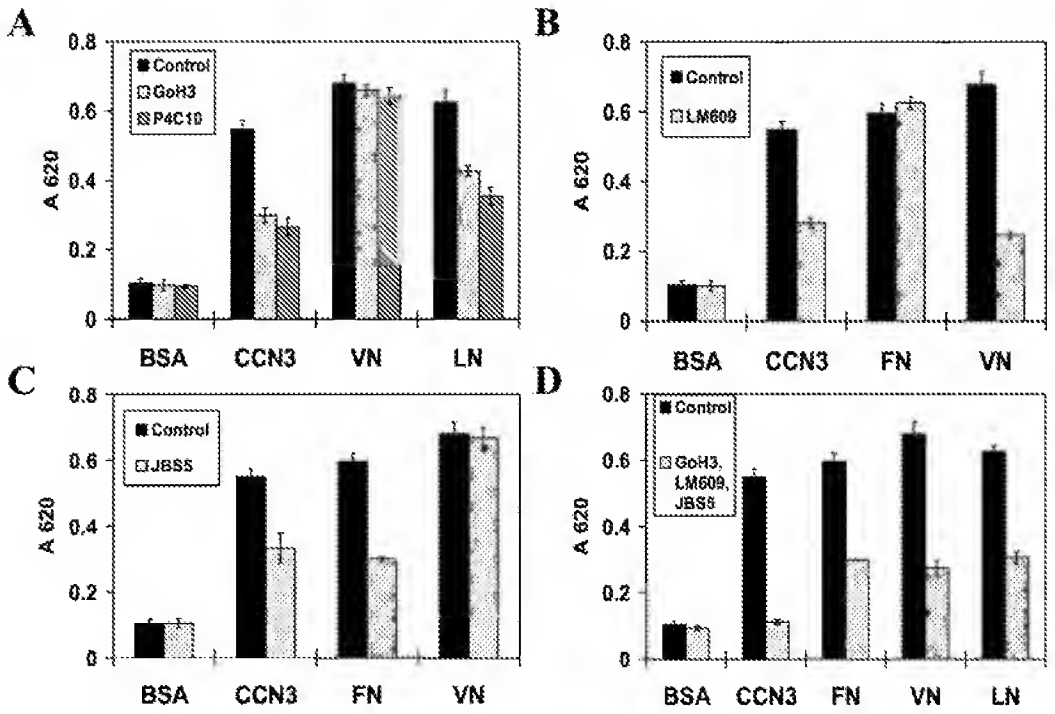

FIG. 3. **HUVEC adhesion to CCN3 is mediated through integrins $\alpha_v\beta_3$, $\alpha_6\beta_1$, and $\alpha_5\beta_1$.** Cell adhesion assays were performed with HUVECs as described in the legend to Fig. 2. *A*, cells were incubated with 40 μg/ml mAbs against integrins α_6 and β_1 for 1 h prior to being plated on microtiter wells coated with 15 μg/ml CCN3, 0.5 μg/ml VN, or 5 μg/ml LN. *B*, cells were incubated with 40 μg/ml LM609 (anti-$\alpha_v\beta_3$ mAb) for 1 h prior to being plated on microtiter wells coated with 15 μg/ml CCN3, 0.25 μg/ml VN, or 3 μg/ml FN. *C*, cells were incubated with 20 μg/ml anti-$\alpha_5\beta_1$ integrin antibody JBS5 for 1 h prior to being plated on microtiter wells coated with 15 μg/ml CCN3, 0.5 μg/ml VN, 1.5 μg/ml FN. *D*, cells were incubated with a combination of mAbs against integrins α_6 (GoH3, 20 μg/ml), $\alpha_v\beta_3$ (LM609, 20 μg/ml) and $\alpha_5\beta_3$ (JBS5, 10 μg/ml) for 1 h prior to being plated on microtiter wells coated with 15 μg/ml CCN3, 0.25 μg/ml VN, 5 μg/ml LN, and 1.5 μg/ml FN. Data shown are mean ± S.D. of triplicate determinations and are representative of three experiments.

not the control GRGESP peptide, was able to partially block cell adhesion to CCN3 and completely block adhesion to VN (Fig. 2*D*), indicating the involvement of RGD-sensitive integrins. Since integrin $\alpha_v\beta_3$ is known to be inhibited by a relatively low concentration of RGD peptide (0.2 mM) as shown in Fig. 2*D*, we examined the role of this integrin. Cells were incubated with LM609, a mAb against integrin $\alpha_v\beta_3$, prior to plating. LM609 partially blocked HUVEC adhesion to CCN3 and VN but not to FN (Fig. 3*B*). We also tested the possible involvement of integrin $\alpha_5\beta_1$ given its RGD sensitivity, even though no other CCN protein is known to bind this integrin. To our surprise, mAb against integrin $\alpha_5\beta_1$ (JSB5) was also able to partially block HUVEC adhesion to CCN3 and FN (Fig. 3*C*). As expected, the same mAb had no effect on HUVEC adhesion to VN. These results indicate that both integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ play a role in HUVEC adhesion to CCN3.

Since it was previously shown that CCN1 and CCN2 mediate adhesion of fibroblasts through integrin $\alpha_6\beta_1$ and heparan sulfate proteoglycans acting as co-receptors (8, 20), we investigated whether these two receptors also mediate HUVEC adhesion to CCN3. To investigate the potential role of integrin $\alpha_6\beta_1$, cells were incubated with mAbs against either integrin α_6 (GoH3) or β_1 (P4C10) subunit prior to plating. These mAbs partially blocked adhesion to CCN3, indicating that integrin $\alpha_6\beta_1$ also plays a role in HUVEC adhesion to CCN3 (Fig. 3*A*). As expected, these mAbs partially blocked cell adhesion to LN, which binds integrins $\alpha_6\beta_1$ and $\alpha_2\beta_1$, but not to VN, which binds α_v integrins. Soluble heparin is known to block fibroblast adhesion to CCN1 and CCN2 by saturating the heparin binding sites located in the CT domain, thereby preventing them from binding cell surface heparan sulfate proteoglycans (8, 20). Likewise, soluble heparin partially blocked HUVEC adhesion to CCN3, but not to FN or VN (Fig. 2*D*). Thus, it is likely that CCN3 may also engage heparan sulfate proteoglycans as a co-receptor when interacting with integrin $\alpha_6\beta_1$.

Given that antagonists of integrins $\alpha_v\beta_3$, $\alpha_6\beta_1$, and $\alpha_5\beta_1$ were able to partially inhibit HUVEC adhesion to CCN3, we tested whether the combination of antagonists of these integrins were sufficient to account for endothelial cell adhesion to CCN3. When cells were treated with GRGDSP peptide and soluble heparin, CCN3 adhesion was obliterated (Fig. 2*D*). In contrast, adhesion of the same cells to FN was not affected. Also, when HUVECs were incubated with function-blocking mAbs against integrins $\alpha_v\beta_3$, α_6, and $\alpha_5\beta_1$, cell adhesion to CCN3 was completely abolished (Fig. 3*D*). As expected, HUVEC adhesion to FN, VN, and laminin was only partially blocked by these antibodies. Together, these results indicate that endothelial cell adhesion to CCN3 is mediated through the combined actions of integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$.

CCN3 Binds Directly to Integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in Vitro—Since CCN3 mediates HUVEC adhesion through integrins $\alpha v\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ (Fig. 3*D*), we investigated whether CCN3 can bind integrin receptors directly. Purified integrins $\alpha_v\beta_3$ or $\alpha_5\beta_1$ were immobilized on microtiter wells, then CCN3 was added in varying concentrations, and binding was detected using anti-CCN3 antibodies. As shown in Figs. 4*A* and 5*A*, CCN3 was able to bind immobilized integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in a dose-dependent and saturable manner, with half-maximal binding occurring at 0.4 μg/ml (10 nM) and 1 μg/ml (50 nM) CCN3, respectively. Conversely, CCN3 was immobilized on microtiter wells and allowed to interact with integrins $\alpha_v\beta_3$ or $\alpha_5\beta_1$. Binding of integrins to immobilized CCN3 was observed using antibodies against integrin α_v or α_5 subunits (Figs. 4, *B–D* and 5, *B–D*).

We employed a variety of antagonists to address the specificity of the interaction between CCN3 and integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. When CCN3 was incubated with affinity-purified anti-CCN3 polyclonal antibodies, binding to integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$

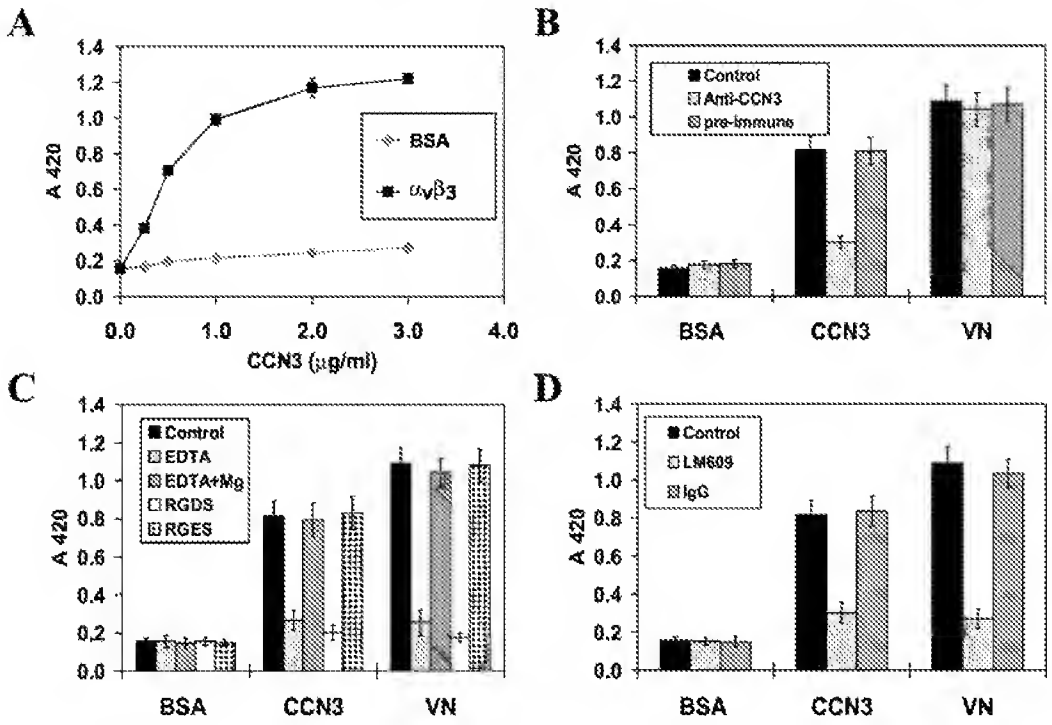

FIG. 4. **CCN3 binds directly to integrin $\alpha_v b_3$.** *A*, microtiter wells were coated with purified integrin $\alpha_v\beta_3$ (1 μg/ml) and blocked with 1% BSA. Binding of varying concentrations of CCN3 was detected using anti-CCN3 antibodies. *B–D*, microtiter wells were coated with CCN3 (10 μg/ml) or VN (1 μg/ml) and blocked with BSA. Effects of preincubation of coated proteins with anti-CCN antibodies (*B*), 20 μg/ml LM609 (*D*), or 20 μg/ml normal mouse IgG (*D*) prior to addition and binding of integrin $\alpha_v\beta_3$ was observed. *C*, integrin $\alpha_v\beta_3$ was incubated with 5 mM EDTA, EDTA + 10 mM Mg^{2+}, 0.2 mM RGDS peptide, or 0.2 mM RGES peptide for 30 min at 4 °C prior to addition into microtiter wells. Binding was detected using anti-α_v antibodies. Data shown are from three separate experiments and represented as mean ± S.D. of duplicate determinations in each experiment.

was abolished (Figs. 4*B* and 5*B*). By contrast, incubation of FN (Fig. 5*B*) or VN (Fig. 4*B*) with the same antibodies had no effect, as expected. Divalent cations are required for integrin function, and both integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ can be inhibited by RGD-containing peptides (41). As shown in Figs. 4*C* and 5*C*, EDTA completely abrogated the binding of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ to CCN3, as well as to VN and FN. As expected, binding was restored upon the addition of $MgCl_2$. RGDS peptide, but not RGES peptide, was able to inhibit binding of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ to CCN3 and to positive controls, VN or FN. Furthermore, mAb against integrin $\alpha_v\beta_3$ (LM609) blocked binding of integrin $\alpha_v\beta_3$ to CCN3 and VN (Fig. 4*D*), and mAb against integrin $\alpha_5\beta_1$ (JBS5) inhibited binding of integrin $\alpha_5\beta_1$ to CCN3 and FN (Fig. 5*D*). Taken together, these results show that CCN3 is a novel ligand of integrins and binds directly and specifically to integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$.

BACECs Migrate to CCN3 through Integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$—Cell migration is an integral part of the angiogenic process, and induction of endothelial cell chemotaxis correlates with angiogenic activity (42). We thus investigated whether CCN3 can stimulate migration of endothelial cells. Using a modified Boyden chamber assay, we found that CCN3 was able to stimulate migration of BACECs (Fig. 6*A*). CCN3-induced migration was dose-dependent and reached maximal level at 0.25 μg/ml. A higher concentration of CCN3 was less effective in inducing cell migration, resulting in a bell-shaped dose response curve observed for many chemotactic factors. Incubation of CCN3 protein with affinity-purified anti-CCN3 antibodies abolished CCN3-induced migration, while incubation with the same antibodies did not affect FN-stimulated migration (Fig. 6*B*), indicating that cell migration can be attributed to CCN3.

Stimulation of cell migration can be due to a chemotactic (directed cell movement) or a chemokinetic (random cell movement) response. In order to determine whether CCN3 induces chemotaxis or chemokinesis, a checkerboard analysis was performed. CCN3 was placed in the upper chamber (no cells), in the lower chamber (with cells), and in both or neither chambers (Fig. 6*C*). Addition of CCN3 to the lower chamber did not enhance BACEC migration to the upper chamber, indicating that CCN3 did not induce a chemokinetic response. Addition of CCN3 to the upper chamber induced the maximal level of migration, consistent with a chemotaxis. Addition of CCN3 to both chambers reduced the level of BACEC migration, suggesting that BACECs are sensitive to a CCN3 gradient. Together, these results show that CCN3 induces directed endothelial cell migration.

Since we have shown that CCN3 is a ligand for integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, both of which can mediate cell migration, we tested whether CCN3 promotes BACEC migration through these integrins. Incubation of cells with GRGDSP peptide completely inhibited migration to CCN3 and VN (Fig. 6*D*). No effect on migration was observed with cells incubated with GRGESP control peptide. Consistent with the involvement of integrin $\alpha_v\beta_3$, anti-integrin α_v mAbs (AV1), and anti-integrin $\alpha_v\beta_3$ mAbs (LM609) partially block BACEC migration to CCN3 (Fig. 7*A*). As expected, these antibodies inhibited BACEC migration to VN but had no effect on cell migration to FN. In addition, anti-integrin α_5 mAb (SAM-1) partially inhibited CCN3-stimulated cell migration but not VN-stimulated cell migration (Fig. 7*B*), indicating that integrin $\alpha_5\beta_1$ may also be involved. Moreover, a combination of mAbs against integrin $\alpha_v\beta_3$ and α_5 mAb (LM609 and SAM-1) completely abolished BACEC migration to CCN3. As expected, these mAbs partially inhibited cell migration to VN and FN and had no effect on cell migration to laminin (Fig. 7*C*). To address whether integrin $\alpha_6\beta_1$ might also play a role, we examined the inhibitory activity of the anti-α_6 mAb, GoH3 (Fig. 7*D*). BACEC migration to CCN3 or FN was unaffected by the presence of GoH3, whereas cell migration to

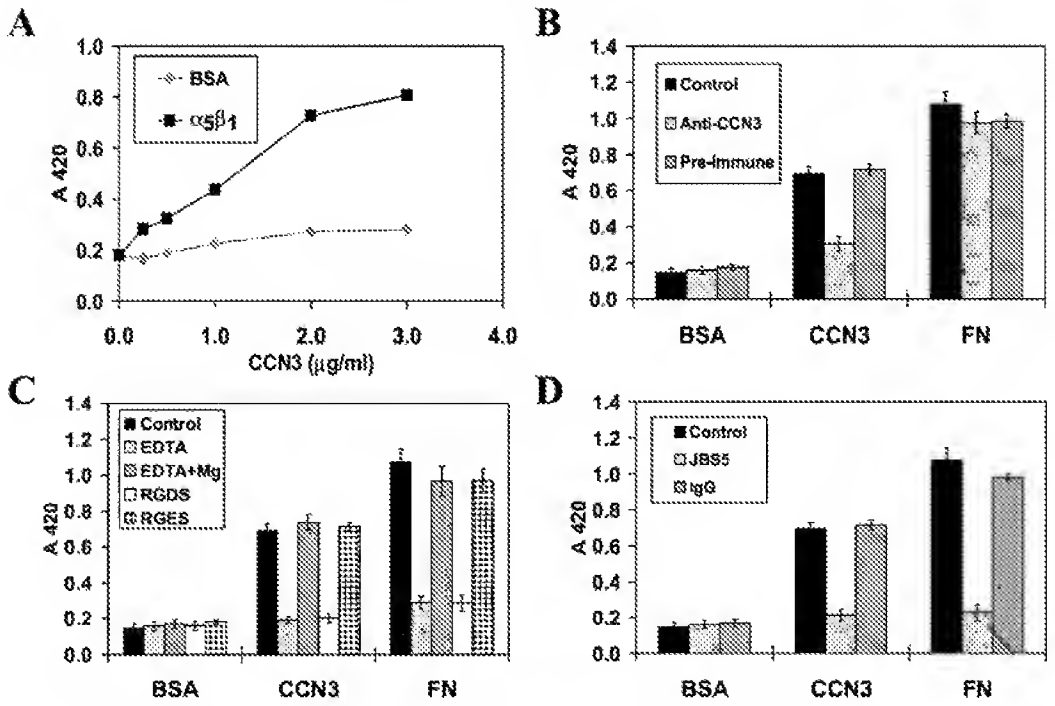

FIG. 5. **CCN3 binds directly to integrin $\alpha_5\beta_1$.** *A*, microtiter wells were coated with purified integrin $\alpha_5\beta_1$ (1 μg/ml) and blocked with 1% BSA. Binding of varying concentrations of CCN3 was detected using anti-CCN3 antibodies. *B–D*, microtiter wells were coated with CCN3 (10 μg/ml) or FN (10 μg/ml) and blocked with BSA. Effects of preincubation of coated proteins with anti-CCN antibodies (*B*), 20 μg/ml JBS5 (*D*), or 20 μg/ml normal mouse IgG (*D*) prior to binding of integrin $\alpha_5\beta_1$ was observed. *C*, integrin $\alpha_5\beta_1$ was incubated with 5 mM EDTA, EDTA + 10 mM Mg^{2+}, 2.0 mM RGDS peptide, or 2.0 mM RGES peptide for 30 min at 4 °C prior to addition into microtiter wells. Binding was detected using either anti-β1 (*B*) or anti-α_5 (*C* and *D*) antibodies. Data shown are from three separate experiments and represented as mean ± S.D. of duplicates.

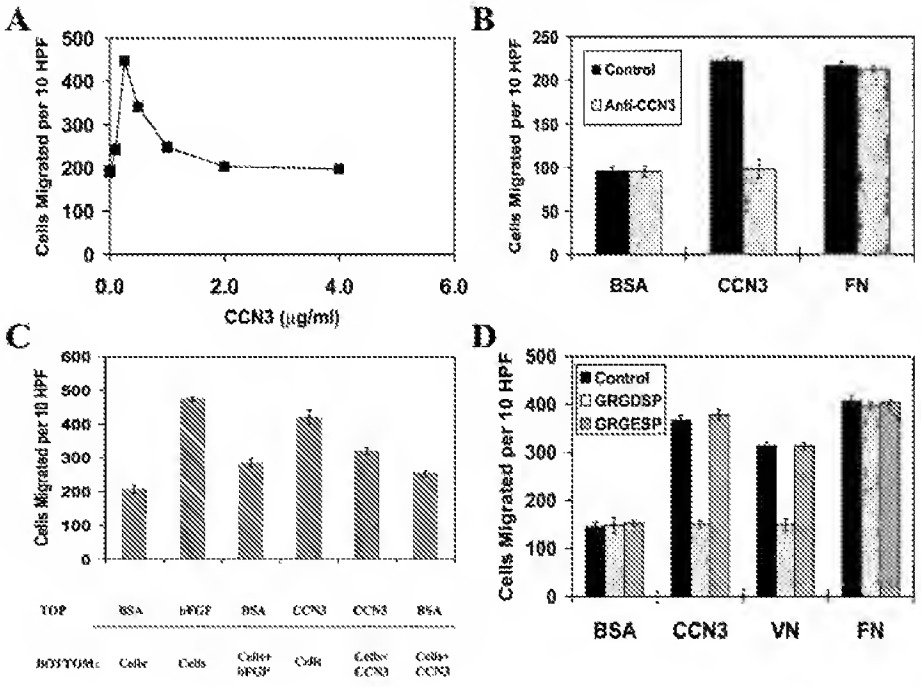

FIG. 6. **Migration of BACEC to CCN3.** BACEC migration was monitored using a modified Boyden chamber assay. Cells were added to wells in the lower chamber and allowed to attach to the gelatinized polycarbonate filter. bFGF (10 ng/ml), CCN3 (0.25 μg/ml unless otherwise indicated), VN (10 μg/ml), or FN (10 μg/ml) were placed in the top chamber (unless otherwise indicated). Cells were allowed to migrate for 4 h at 37 °C before being fixed and stained, and cells that migrated into the upper chamber were counted in ten random high power fields. Cell migration in response to varying concentrations of CCN3 (*A*), the presence of anti-CCN3 antibodies (*B*), or 0.2 mM GRGDSP or GRDESP peptides (*D*) was assessed. *C*, the migration of BACEC was measured in a checkerboard-type analysis. CCN3 or bFGF were added to the upper chamber, the lower chamber, neither chamber, or both chambers as indicated. Data shown are mean ± S.D. of triplicate determinations and are representative of three experiments.

laminin was partially inhibited. Taken together, these results show that of the three integrins known to interact with CCN3 (Figs. 2–5), integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ mediate endothelial cell migration to CCN3, whereas integrin $\alpha_6\beta_1$ is not involved in this process.

CCN3 Promotes Endothelial Cell Survival—During angiogenesis, endothelial cells require survival signals in order to migrate, proliferate, and interact with the remodeling ECM. Ligation of integrin $\alpha_v\beta_3$ has been shown to induce survival signals in endothelial cells (43). To investigate the possibility that CCN3 may promote cell survival, HUVECs were plated on laminin and maintained in serum-free medium. Under these conditions, endothelial cells were susceptible to apoptosis (44). GRGDSP peptide, 20% serum, or varying concentrations of CCN3 were then added to cells, and apoptosis was determined using a TUNEL assay after 16 h (Fig. 8*A*). Under these conditions, CCN3 was able to promote endothelial cell survival in a dose-dependent manner. Serum also protected cells from apoptosis, while GRGDSP peptide promoted apoptosis. Preincubation of CCN3 with affinity-purified anti-CCN3 antibodies abol-

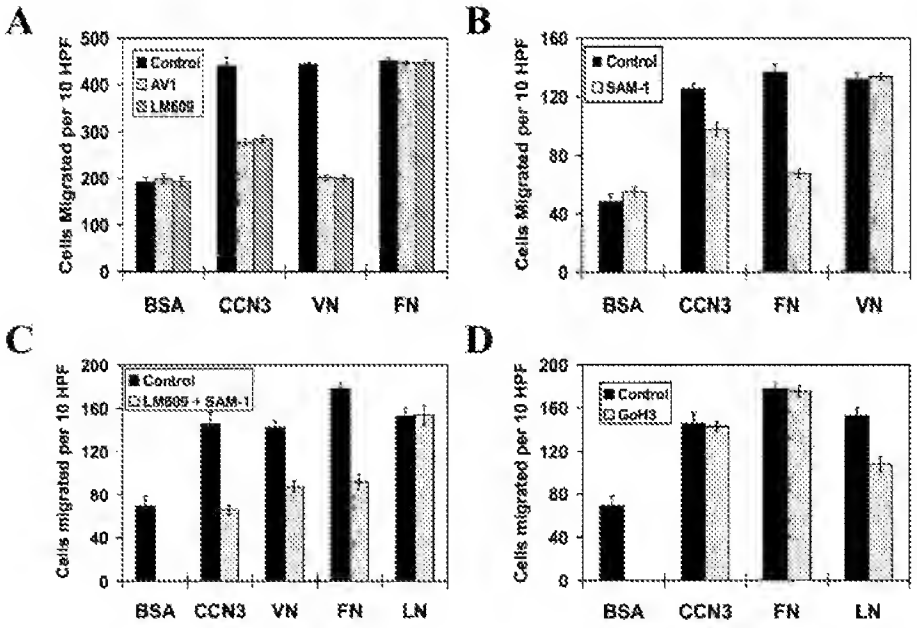

FIG. 7. **Integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ mediate migration of BACEC to CCN3.** Migration assays were performed using a modified boyden chamber. As chemoattractants, CCN3 (0.25 μg/ml), vitronectin (10 μg/ml), and FN (10 μg/ml) were placed in the top chamber. *A*, cells were treated with anti- integrin α_v (AV1, 60 μg/ml) or anti-integrin $\alpha_v\beta_3$ (LM609, 60 μg/ml) for 1 h prior to chamber loading. Cells were also preincubated with either anti-integrin α_5 (*SAM-1*, 40 μg/ml)(*B*), both SAM-1 (12.5 μg/ml) and LM609 (25 μg/ml) (*C*), or anti-integrin α_6 (GoH3, 25 μg/ml) (*D*) for 1 h prior to chamber loading. Data shown are mean ± S.D. of triplicate determinations and are representative of three experiments.

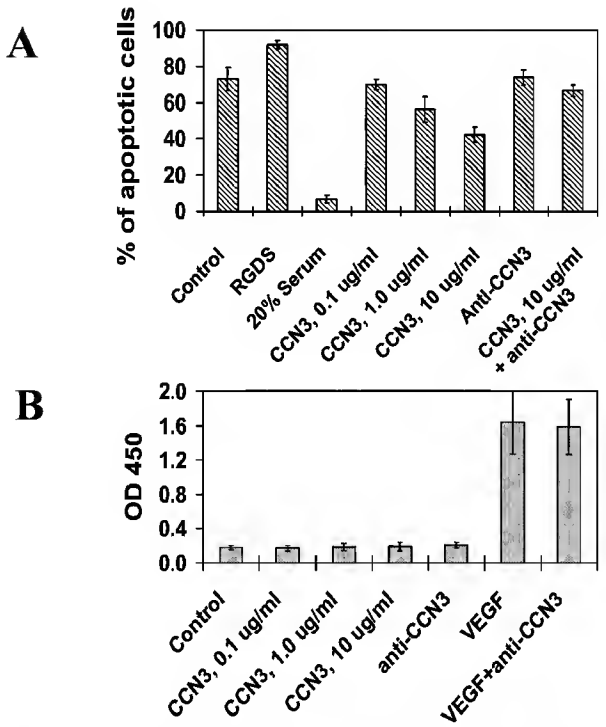

FIG. 8. **CCN3 protects HUVECs from apoptosis.** HUVECs were serum-starved prior to attachment to coverslips pre-coated with 20 μg/ml LN. *A*, cells were then incubated in serum-free medium for 4 h. This was followed by addition of serum, various concentrations of CCN3 protein or 50 μM GRGDSP peptide and incubation at 37 °C for an additional 16 h. Cells were fixed and apoptosis was monitored by using a TUNEL assay; the number of apoptotic cells were then counted. In the last bar, 10 μg/ml CCN3 was preincubated with anti-CCN3 antibodies prior to addition into the medium. *B*, HUVECs treated as described in *panel A* were labeled with BrdUrd for 16 h, and cells incorporating label in the absence or presence of CCN3 are quantified by colorimetric determination. Data shown are mean ± S.D. of triplicate determinations and are representative of three experiments.

ished CCN3-promoted cell survival. This was not due to antibody toxicity since addition of antibodies alone did not affect cell survival. Taken together, these results show that CCN3 can protect endothelial cells from apoptosis and promote survival. To rule out the possibility that an increased number of non-apoptotic cells might be due to cell proliferation, HUVECs were treated in the same manner as described above and proliferation was measured using BrdUrd incorporation (Fig. 8*B*). The rate of DNA synthesis was not affected by the presence or absence of CCN3. These results show that CCN3 is not a mitogen for HUVECs under these conditions, and that CCN3 is able to promote endothelial cell survival under otherwise apoptotic conditions.

CCN3 Induces Neovascularization in Vivo—The ability of CCN3 to promote endothelial cell adhesion, migration, and survival are consistent with properties of an angiogenic inducer. We therefore investigated whether CCN3 could induce angiogenesis *in vivo* using the rat corneal micropocket assay. As shown in Fig. 9, CCN3 was able to induce neovascularization when implanted into rat cornea, whereas the vehicle did not induce any response (Table I). Neovascularization was also observed in corneas implanted with Hydron pellets containing bFGF, a known potent angiogenic inducer. Preincubation of CCN3 with anti-CCN3 antibodies obliterated CCN3-induced neovascularization, indicating that the angiogenic activities observed can be ascribed to the CCN3 polypeptide. Together, these results show that CCN3 can induce angiogenesis *in vivo*.

DISCUSSION

Although CCN3 was first identified more than 10 years ago, little is known about its biochemical activities and biological functions. In this study, we show that purified CCN3 is capable of pro-angiogenic activities in endothelial cells. CCN3 supports endothelial cell adhesion, stimulates directed cell migration, and promotes cell survival. Furthermore, CCN3 induces neovascularization *in vivo* in a corneal micropocket assay. Mechanistically, CCN3 acts as a ligand of integrin receptors and mediates endothelial cell adhesion through integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, whereas its chemotactic activity is mediated through integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Despite lacking a RGD sequence motif, CCN3 binds directly to integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. These findings establish CCN3 as a novel integrin ligand and angiogenic inducer, provide insights into its mechanism of action, and suggest biological functions for CCN3 both in normal development and in pathological conditions where its aberrant expression has been observed.

Angiogenesis, or the formation of new blood vessels from pre-existing ones, is a complex process requiring the coordinated execution of multiple cellular events (45). The sprouting of vessels requires degradation of the basement membrane

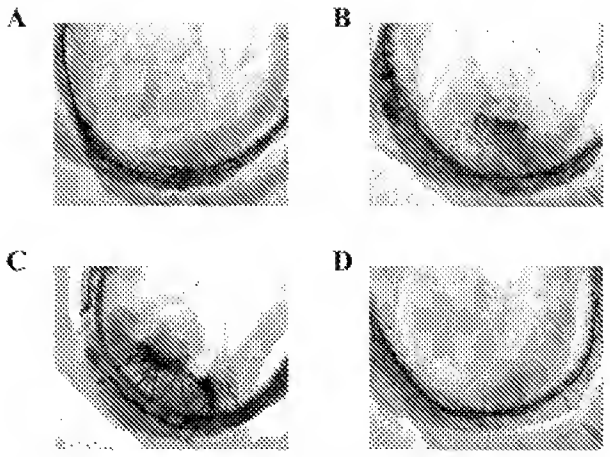

FIG. 9. **CCN3 induces neovascularization in rat corneas.** Hydron pellets containing test substances were made and implanted into rats corneas (Table I). Blood vessel formation was visualized by perfusion with colloidal carbon 7 days after implantation. Vessel formation due to Hydron pellets containing CCN3 storage buffer (*A*), bFGF (*B*), CCN3 protein (*C*), and CCN3 protein preincubated with anti-CCN3 antibodies (*D*), are shown.

TABLE I
Effects of CCN3 on corneal neovascularization

Hydron pellets containing CCN3 storage buffer, CCN3 (300 ng), bFGF (50 ng), or CCN3 preincubated with anti-CCN3 antibodies (400 ng) were implanted into rat corneas. Corneal vascularization was scored after 7 days.

Test substance	Vascularized (+) and unvascularized (−) corneas	
	+	−
CCN3	13	1
bFGF	7	1
CCN3 buffer	0	7
CCN3 + anti-CCN3 antibodies	0	8

surrounding the parental vessel, migration of vascular endothelial cells toward the angiogenic stimulus, proliferation of endothelial cells and their alignment into tubular structures, and coalescence of new vessels into circular loops to provide blood supply to the target tissue (45). Angiogenesis is essential for embryogenesis, and in the adult, it is important in the female reproductive cycle and in wound healing. Angiogenesis may underlie a number of pathological conditions including diabetic retinopathy, arthritis, atherosclerosis, psoriasis, and cancer (46). It is now clear that angiogenesis is regulated by a network of multiple inducers and inhibitors (47, 48), and CCN3 may be part of this network of regulators. Our conclusion that CCN3 is a novel angiogenic inducer is based on the observations that it acts directly upon endothelial cells to promote cell adhesion, migration, and cell survival *in vitro* (Figs. 2, 6, and 8), and induces angiogenesis *in vivo* (Fig. 9). These activities are inhibited by antibodies specific for CCN3, showing that they are intrinsic properties of the CCN3 polypeptide. In this study, we have employed HUVECs and BACECs to examine the angiogenic functions of CCN3. It is important to note that there is considerable heterogeneity among vascular endothelial cells, differing in morphology and function, matrix environment, and responses to growth factors (49–51). Thus, it is possible that other endothelial cells may exhibit different responses to NOV in culture.

The discovery that CCN3 is an angiogenic factor helps to shed light on its functions in development and disease. CCN3 is expressed in hypertrophic cartilage (26), where vessel growth is required for the formation of a scaffold onto which the osteoblasts settle and deposit bone matrix (52). Thus, CCN3-induced angiogenesis may be important in endochondral ossification. During nephrogenesis, CCN3 is localized to the metanephric mesenchyme into which endothelial cells are recruited (32). These endothelial cells then proliferate and form a capillary network as the metanephric mesenchyme develops to form the glomeruli, the basic units of filtration (53). The presence of CCN3 might help serve as a chemotactic and survival factor for endothelial cells. In addition, CCN3 expression is correlated with various tumors, including Wilm's tumors, and benign adrenocortical tumors (27, 32, 54). It is well established that tumor growth beyond ~1 mm in size requires the growth of new vessels to provide the necessary blood supply (47, 55). Thus, the expression of CCN3 in tumors is consistent with its angiogenic activity. Furthermore, the Wilms' tumor suppressor gene (WT1) was shown to negatively regulate CCN3 expression (56). It is possible to speculate that, as part of its function, WT1 down-regulates the angiogenic inducer CCN3 to help suppress tumor growth.

We have also identified the cell surface receptors that mediate CCN3 functions in endothelial cells. CCN3 supports endothelial cell adhesion through integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, whereas chemotaxis is mediated through $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (Figs. 3 and 7). Furthermore, CCN3 binds directly to integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (Figs. 4 and 5). Although it is well established that integrins are important in developmental and pathological angiogenesis (57), it is currently unclear which integrins play a greater role in CCN3-induced angiogenesis. Interestingly, CCN3 is the only member of the CCN protein family known to bind integrin $\alpha_5\beta_1$ to date. By contrast, integrin $\alpha_5\beta_1$ plays no role in CCN1-mediated endothelial cell adhesion (7), and we have found that CCN1 does not bind purified $\alpha_5\beta_1$ in a solid phase binding assay (data not shown). The role of integrin $\alpha_5\beta_1$ in developmental angiogenesis has been established genetically in mice, where targeted gene disruptions in integrins α_5 or β_1 resulted in embryonic lethality with prominent angiogenic defects (58–60). Inasmuch as CCN3 acts directly on endothelial cells through integrin $\alpha_5\beta_1$ to promote cell adhesion and migration, the binding of CCN3 to $\alpha_5\beta_1$ may be critical to CCN3-mediated angiogenesis.

A wealth of data also supports the notion that integrin $\alpha_v\beta_3$ plays a critical role in angiogenesis. Importantly, antagonists of integrin $\alpha_v\beta_3$ effectively block angiogenesis both *in vitro* and *in vivo*, and inhibit tumor formation in animal models (61, 62). A humanized monoclonal antibody against integrin $\alpha_v\beta_3$, Vitaxin, is currently undergoing clinical trial as an anti-cancer drug (63). This therapeutic approach is predicated on the premise that integrin $\alpha_v\beta_3$ acts as a pivotal regulator of angiogenesis. However, recent studies have raised questions challenging this view (64). Human or mice deficient in the integrin β_3 subunit display normal developmental angiogenesis and are viable and fertile, although they have a bleeding disorder due to defects in the platelet integrin $\alpha_{IIb}\beta_3$ (65). This finding indicates that $\alpha_v\beta_3$ is not absolutely required for developmental angiogenesis. Furthermore, mice with targeted disruptions in integrin β_3 or both β_3 and β_5 subunits grow larger tumors than wild type, suggesting that integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ may actually be negative regulators of angiogenesis (66). These observations indicate that the roles of α_v integrins in angiogenesis may be more complex than previously thought. Nevertheless, the ability of CCN3 to bind integrin $\alpha_v\beta_3$ and to promote endothelial cell adhesion and migration through this integrin shows that angiogenic actions may be mediated through $\alpha_v\beta_3$. In this context, it is possible to contemplate the actions of CCN3 both as an inducer via direct binding to $\alpha_v\beta_3$, and as a modulator by competition with other ligands that bind integrin $\alpha_v\beta_3$.

Members of the CCN family of matricellular proteins clearly

serve important developmental functions. Deficiency in CCN6 causes progressive pseudorheumatoid dysplasia in humans, a juvenile-onset degenerative disease of the cartilage (67). Although both CCN1 and CCN2 are angiogenic inducers, mutations in their structural genes result in distinct phenotypes related to angiogenic defects. Targeted gene disruption of *CCN1* in mice resulted in embryonic lethality with vascular defects in both the placenta and the embryo (23). *CCN2*-null mice, on the other hand, are perinatal lethal as a consequence of respiratory failure due to skeletal malformations (18). Interestingly, angiogenic defects in the growth plates underlie part of the skeletal defects in *CCN2* mutants. These findings indicate that although proteins of the CCN family share extensive sequence homology and overlapping activities *in vitro*, they serve non-redundant developmental functions. Thus, although CCN3 induces angiogenesis, its biological roles may be distinct from those of CCN1 and CCN2. The observation that CCN3 is the only CCN protein known to bind integrin $\alpha_5\beta_1$ also supports the notion that CCN3 has unique functions. Analysis of CCN3 mutants in mice will be most informative in revealing its developmental and pathological roles.

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